NUCLEAR MYOSIN I ß WITH A 16 AMINO ACID N-TERMINAL EXTENSION

INVENTORS: Primal de Lanerolle, Grzegorz Nowak, Lidija Pestic-Dragovich, Ljuba Stojiljkovic and Pavel Hozak

BACKGROUND OF THE INVENTION

The present invention claims priority from co-pending U.S. Serial No. 60/214,944 filed June 29, 2000.

The U.S. Government may have rights in the present invention due to partial support provided by NSF MCB 9631833, NSF INT 972416, and NIH HL 59618, NIH GM 37537 and NSF Program ME143.

The present invention is directed to a molecular motor actin binding protein, designated Nuclear Myosin I ß (NMI ß), that includes a 16 amino acid N-terminal extension not present in in other myosin proteins. The 16 amino acid extension is unique to nuclear myosin I ß and is responsible for the nuclear localization of this protein.

Myosin I is a member of a superfamily of actin binding proteins that hydrolyze adenosine triphosphate (ATP) (Mermall *et al.*, 1998). Most members of this superfamily have been identified based on their DNA sequences. Myosin II is the prototypic member of this family. Myosin II is a two headed, filamentous protein that is an actin-activated ATPase. Myosin II is widely distributed in eukaryotic cells and its role in energy conversion during muscle contraction is well known.

Myosin I is a single headed, non-filamentous, actin-activated ATPase (Pollard and Korn, 1973). First described in *Acanthamoeba castellanii* (Pollard and Korn, 1973), myosin I is widely distributed in metazoan cells (Mermall *et al.*, 1998). There are at least four different subclasses of myosin I proteins, all containing a 110-150 kD heavy chain and 1-6 light chains located in the neck region between the head and tail. In vertebrate forms of myosin I, this light chain is calmodulin.

Immunofluorescence studies of mammalian cells have shown that myosin I is diffusely distributed throughout the entire cytoplasm and that it concentrates near cortical surfaces and in the perinuclear region. Although evidence of specific roles of myosin protein in metazoan cells is lacking, it has been suggested, based on localization studies, that myosin I proteins are molecular motors involved in plasma

membrane extension, vesicle and organelle and mechanochemical regulation of calcium channels in hair cells (Mermall *et al.*, 1998).

The nucleus contains a filamentous network that is analogous to the cytoskeleton, and nascent DNA has been shown to tightly associate with the nuclear "matrix" (Berezney and Coffey, 1975). Although the composition of this matrix or nucleoskeleton is unclear, it has been suggested that replication of DNA occurs as templates move through replication "factories" anchored to the nucleoskeleton (Cook, 1991). Transcription of ribosomal genes takes place in specific locations in nucleoli known as fibrillar centers. Templates move through an array of RNA polymerases on the surface of those fibrillar centers that are part of a dynamic model of transcription.

Actin is a protein that is abundant in the nucleus (Rando *et al.*, 2000) and is frequently found in association with the nuclear matrix. Actin is reported to associate with small ribonucleoproteins in the processing and transport of RNA. Nuclear-specific actin binding proteins also have been described. Because the injection of anti-actin antibodies into *Xenopus* oocytes blocks chromosome condensation and the transcription of lampbrush chromosomes (Scheer *et al.*, 1984), actin may be involved in nuclear movements, transcription or other events that require energy. Actin usually works in concert with myosin. Although the presence of myosin II-like proteins in the nucleus has been suggested, these proteins have not been purified or characterized.

Transcription is a process of constructing messenger RNA (mRNA) molecules using a DNA molecule as a template that results in transfer of genetic information to mRNA. Transcription requires energy, and RNA polymerases have been suggested to power the movement of the transcription complexes in a manner analogous to energy conversion by myosin (Yin *et al.*, 1995). However, the precise mechanism and the molecules involved in energy conversion during transcription are not known.

There is a particular need for cellular molecules or molecular structures that can be used to design target pharmaceuticals, as well as a need for the sequences that encode these molecules. Any protein involved in transcription is a site for therapeutic intervention in proliferative disorders.

An actin-based molecular motor of the myosin superfamily is a candidate as an energy conversion molecule involved in transcription.

SUMMARY OF THE INVENTION

The present invention is directed to a protein designated Nuclear Myosin I ß and the corresponding oligonucleotide sequence. It is a new protein that contains a unique N-terminal sequence, and is a separate gene product that belongs to the myosin I ß family. The myosin superfamily is composed of at least fourteen members including myosin I and myosin II. The myosin I family includes the myosin I ß form. Both the known cytoplasmic and the newly discovered nuclear forms of this protein are members of the myosin I subfamily of the myosin superfamily of proteins.

The invention is a myosin-like protein in the nucleus. Special techniques, including the use of a unique antibody and partial denaturation followed by renaturation prior to immunoprecipitation, were used to isolate myosin Iβ, a member of the myosin superfamily, from the nucleus and to show that it is required for transcription.

It was unexpectedly discovered that the 120 kD protein is a new isoform of myosin I β that has 98% amino acid sequence homology to the previously known cytoplasmic myosin I β, but its functionality is determined by a newly sequenced characteristic 16 amino acid N-terminal extension that is not found in cytoplasmic myosin I.

There has been no previous suggestion of a myosin I β protein containing a 16 amino acid N-terminal extension or that this 16 amino acid extension causes nuclear localization of that protein or that myosin I β is included in the transcription process.

Ins A1

The Nuclear Myosin I β protein has an amino acid sequence shown in FIG. 1 (GenBank Accession Number AY 007255). This amino acid sequence includes an initiator methionine and a 16 amino acid peptide N-terminal to the initiator methionine. The peptide includes the amino acid sequence MRYR ASAL GSDG VRVT at the N-terminal end.

In an embodiment of the present invention, a complementary DNA molecule (cDNA) is synthesized from isolated mRNA's of the Nuclear Myosin I β (NMIβ) 16 amino acid N-terminal extension (FIG. 2).

Ins A1

In another embodiment of the present invention, antibodies are directed to the Nuclear Myosin I β protein. Additionally, antibodies directed to the peptide comprising the amino acid sequence MRYR ASAL GSDG VRVT are produced. The

Ins A1

antibodies directed to Nuclear Myosin I β protein or to the 16 amino acid peptide as described herein may also be monoclonal antibodies.

In another embodiment of the present invention, electron and confocal microscopy revealed that Nuclear Myosin I β co-localizes with RNA polymerase II. RNA polymerase II and Nuclear Myosin I were also shown to co-precipitate, demonstrating that RNA polymerase II and Nuclear Myosin I bind to each other to form a complex.

In another embodiment of the present invention, the Nuclear Myosin Iβ protein forms a functional complex with RNA polymerase II. This complex is actively involved in the transcription process that can be disrupted by inactivating Nuclear Myosin I. The ability to suppress or disrupt the transcription process is useful in designing treatments and gene therapies for conditions wherein cell proliferation should be inhibited.

In an embodiment of the present invention a method is provided for inhibiting cell proliferation. The method comprises the steps of obtaining antibodies to the 16 amino acid N-terminal extension of the Nuclear Myosin I β protein and then administering the antibodies to an organism wherein the antibodies contact cells and may inhibit transcription. The antibodies of this method include monoclonal antibodies.

In another embodiment of the present invention, a screening method for inhibiting transcription is disclosed.

DEFINITIONS AND ABBREVIATIONS USED HEREIN

NMI β = nuclear myosin I β.

CMI β = cytoplasmic myosin I β (ie: NMI β without its characteristic 16 amino acid N-terminal extension).

Molecular motors = molecules that hydrolyze nucleotides and use the energy that is released to do mechanical work at the molecular level.

5'UTR = the untranslated region at the 5 prime end of a nucleotide sequence.

5'RACE = rapid amplification of cDNA ends.

PBS = phosphate buffered saline.

Isoforms = multiple forms of the same protein that differ in their amino acid sequences and are produced by different genes or separate transcripts.

kD = kilodalton.

DTT = dithiothreitol.

EDTA = ethylenediaminetetracetic acid.

EGTA = ethylene glycol-bis(β-amino ethyl ether).

PMSF = phenylmethylsulfonyl fluoride.

BrUTP = 5-bromouridine 5'-triphosphate.

ATP = adenosine 5'-triphosphate.

CTP = cytidine 5'-triphosphate.

GTP = guanosine 5'-triphosphate.

UTP = uridine 5'-triphosphate.

The single letter code for amino acids is used herein.

Amino acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y

Valine	Val	V
unknown or other	Xaa	X

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the amino acid sequence of the 120 kD protein of the present invention. The sequence in bold N-terminal to the initiator methionine shows a peptide not present in the known mouse sequences. The new sequence was obtained by microsequencing and 5'- RACE PCR. The consensus ATP binding site is in brackets and the actin binding site is shown in italics. The peptides that were identified by microsequencing are underlined.

FIG. 2 shows the oligonucleotide sequence for Nuclear Myosin I β.

Ins A1

FIG. 3 lists the 5' region of mouse NMI β cDNA (Panel A) and the organization of the myosin I ß gene on chromosome 11 (Panel B); in Panel A, the two ATG codons corresponding to the consensus start site and NMI β start site are underlined; the peptide that overlaps the consensus start site that was obtained by microsequencing (ASALGSDGVRVTMESALTAR) is shown in bold; the previously known mouse myosin I β cDNA and protein sequences are in italics.

FIG. 4 shows functional association of NMI β and RNA polymerase II.

DETAILED DESCRIPTION OF THE INVENTION

A molecular motor actin binding protein, Nuclear Myosin I β, containing a 16 amino acid N-terminal extension, is provided. The protein of the present invention is a molecule with an oligonucleotide sequence coding for Nuclear Myosin I β. It is a protein with an estimated molecular weight of 120,000 daltons. Microsequencing of the 120 kD protein and 5' RACE PCR showed that the 120 kD nuclear protein is similar to cytoplasmic myosin I β. Surprisingly, the 120 kD protein contained a unique N-terminal extension. This 120 kD protein was named Nuclear Myosin I β (NMI β) to distinguish it from the smaller (116 kD) cytoplasmic myosin I β (CMI β).

As the data from the experiments described herein show, the molecule with an oligonucleotide sequence coding for a Nuclear Myosin I β protein contains an ATP binding site, an actin binding site and a unique peptide sequence at the N-terminal to the initiator methionine on cytoplasmic myosin I β (CMI β).

NMI β is located at sites of actively transcribing genes and it co-localizes with actin in nucleolar structures where ribosomal DNA transcription takes place. The co-

localization of actin and Nuclear Myosin I β in the same nuclear structure suggests that the NMI β could power nuclear processes by binding to actin through the actin binding site on the head and negatively charged nuclear components through a conserved, positively charged tail domain. The protein is closely associated with DNA transcription via its interaction with RNA polymerase II. Nuclear Myosin I β forms functional complexes with RNA polymerase II. The co-localization of Nuclear Myosin I β and RNA polymerase II and the inhibition of RNA synthesis by the anti-NMI β peptide antibodies suggested that Nuclear Myosin I β, perhaps together with RNA polymerases, powers the movement of transcriptional complexes. Transcription is essential for cell proliferation, therefore, any protein that is involved in transcription is a potential target for pharmacological or genetic inhibition of transcription, and consequently of cell proliferation. Because rapid transcription is essential to cell proliferation and cancer is an example of an uncontrolled cell proliferation, Nuclear Myosin I β will be useful to the pharmaceutical industry in developing drugs for disease associated with abnormal cell proliferation.

This invention is also directed to purifying antibodies to the NMI β 16 amino acid N-terminal extension sequence or to an epitope within that sequence, and then administering these antibodies to patients. Given the function of myosin generally as a molecular motor, inactivating NMI β repressed transcription, possibly by preventing the movement of the RNA polymerase II holoenzyme. Thus, a method for suppressing transcription in proliferating cells would include repressing the expression or function of NMI β with targeted pharmaceuticals or gene therapy. In particular, the unique 16 amino acid N-terminal and its DNA sequence is useful for designing treatment and gene therapy.

A. CHARACTERIZATION OF THE NUCLEAR MYOSIN I PROTEIN

1. Immunological Studies

a. Fluorescence, Confocal and Electron Microscopy

<u>Procedure</u>: Light microscopy was performed on cells growing on coverslips. The cells were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) and permeabilized with 0.1% deoxycholic acid and 0.1% Triton X-100 in PBS. The cells were then stained with primary antibodies for 2 hrs. at room temperature and visualized using a Texas red, rhodamine or fluorescein conjugated

secondary antibodies Coverslips were mounted using Vectashield with DAPI and cells were photographed on a Zeiss Laser confocal microscope.

Electron microscopy was performed on cells grown at 37°C in suspension in Eagle medium containing 5% fetal bovine serum. Cells were pelleted, fixed in 3% paraformaldehyde plus 0.1% glutaraldehyde in Sörensen buffer, embedded and sectioned using methods known to those of skill in the art. Eighty nanometer thick sections were incubated simultaneously with the rabbit polyclonal antibody to adrenal myosin I (3 μg/ml) and a monoclonal anti-actin antibody (5 μg/ml, Amersham Corporation), washed and incubated with 5 nm gold-conjugated goat anti-mouse antibody and with 10 nm gold-conjugated goat anti-rabbit (British Biocell). After washing, the sections were contrasted for 5 min with a saturated solution of uranyl acetate in water and observed using a Phillips cm. 100 electron microscope.

Results: Epifluorescence and confocal microscopy using the polyclonal antibodies to myosin I showed myosin I β staining in the perinuclear region and the leading edges of migrating cells (Nowak *et al.*, 1997). These antibodies also stained the nucleus. Optically sectioning cells through the nucleus demonstrated intranuclear staining.

Electron microscopy demonstrated the presence of immunoreactive material in the cytoplasm and the nucleoplasm of 3T3 cells. (Nowak *et al.*, 1997) Micrographs showed the localization of myosin I β antibodies with strong labeling in the nucleoplasm. Moreover, the protein recognized by the myosin I β antibody co-localized with actin in the nucleus. High power views through a nucleolus showed that the myosin I β antibodies were found mainly in the "dense fibrillar component" where rDNA transcription occurs. These structures surrounded fibrillar centers that contained actin.

b. Western Blot Analysis of Nuclear and Cytoplasmic Fractions

Procedure: Myosin I β was purified from bovine adrenal glands as described (Barylko *et al.*, 1992) and then polyclonal antibodies to bovine adrenal myosin I were raised in rabbits using a technique known to those of skill in the art. Specific antibodies were purified from the immune serum on an adrenal

myosin I β-Sepharose 4B column. Purified adrenal myosin I, whole cell extracts and cytoplasmic and nuclear fractions were separated by SDS-PAGE and transferred to nitrocellulose paper. The nitrocellulose sheets were incubated with affinity-purified polyclonal antibodies to adrenal myosin I (0.4 μg/ml) or mouse monoclonal anti-myosin I β antibodies (10 μg/ml) followed by peroxidase-conjugated secondary antibodies.

Results: The well characterized M2 monoclonal antibody to adrenal myosin I β (Barylko *et al.*, 1992) cross-reacted with purified adrenal myosin I and a cytoplasmic protein that co-migrated with adrenal myosin I. This protein has a molecular weight of 116 kD. The polyclonal antibodies to adrenal myosin I also recognized purified adrenal myosin I and a 116 kD cytoplasmic protein that co-migrated with adrenal myosin I. In addition, the polyclonal antibodies to adrenal myosin I also recognized proteins with a slightly higher molecular weight (Mr = 120,000) in whole cell extracts and nuclear fractions prepared from a number of mammalian cells. The difference in the molecular weight of the myosin I β proteins identified by the two antibodies was confirmed by reprobing the nitrocellulose sheets with the complementary antibodies, i.e. first polyclonal antibody and then the monoclonal antibody and vice versa. Thus, the 116 kD protein appeared to be present only in the cytoplasm while the 120 kD protein was found in both the cytoplasm and the nucleus.

2. Determination of the Biochemical Properties of the 120 kD Protein

Procedure: To partially purify the 120 kD protein, the nuclei from about 2 billion cells were isolated using established methods and washed twice by resuspension/centrifugation through a sucrose cushion. The nuclei were resuspended in 1 M NaCl, 1 mM DTT, 0.5% Nonident P-40, 0.4 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM $MgCl_2$, 40 mM DNase, 20 mM Tris-HCl, pH 7.5 at 4°C and extracted by passing through hypodermic needles of decreasing gauge down to a 27 gauge needle. The supernatant was collected by centrifugation (20 mins at 50,000 XG), made 10 mM ATP and subjected to ammonium sulfate fractionation. The protein precipitating between 20 and 65% ammonium sulfate was dissolved in 0.5 M NaCl, 1 mM DTT, 5 mM EGTA, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5. ATP and $MgCl_2$ were added to final concentrations of 10 and 12 mM,

respectively, the solution was clarified by centrifugation as above and applied to 1.25 X 70 cm Sepharose 4B gel filtration column.

Fractions containing the 120 kD protein were pooled, concentrated and analyzed to determine if the 120 kD binds actin. The concentrated column fractions were incubated with 0.2 mg/ml F-actin, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5 (100 μl total) for 60 mins at 4°C. Some samples were made 10 mM ATP, final, and incubated for 10 mins more. All samples were then spun at 100,000 X g for 20 mins. The entire pellet and supernatant fractions were analyzed by Western blotting using polyclonal antibodies to myosin I β.

Results: Analysis of Sepharose 4B column fractions showed that fractions containing the 120 kD protein, recognized by the polyclonal antibodies described, also contain K^+-EDTA ATPase activity. K^+-EDTA ATPase activity is a unique feature of myosin molecules that was used to FIRST identify myosin I β (Pollard and Korn, 1973). . This was the first demonstration of K+-EDTA ATPase activity in the nucleus. The 120 kD protein was also shown to bind actin only when ATP was absent. Because K^+-EDTA ATPase activity and actin binding in the absence of ATP are defining features of the myosin superfamily of these data indicated that the 120 kD protein was a member of the myosin superfamily.

3. Positive Identification of the 120 kD Protein

Procedure: Twice washed nuclei (about 40 million) were incubated for 5 min with DFP and then extracted in buffer containing 0.5 mg/ml DNase, 0.2 mg/ml RNase, 1 mM PMSF, 10 ug/ml, each, of aprotinin, leupeptin, pepstatin. The extracts were then centrifuged at 50,000 X g for 15 min and 900 μl of the supernatants were combined with 100 μl of 10 X immunoprecipitation buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton, 5 mM EDTA, 1 mM PMSF, 1% SDS) and mixed. Myosin I β antibody (20 ug) was added and incubated in a cold room, with shaking, overnight. Protein A-Sepharose (300 ul of 50%) was added and incubated in a cold room for 3 hours. The Sepharose beads were washed extensively with immunoprecipitation buffer and the bound protein was eluted by boiling the Sepharose beads twice in 150 ul of 0.25% SDS, 5 mM DTT, 5 mM Tris at pH 6.8. The eluants were combined, lyophilized and resuspended in 10% sucrose, 0.01% bromophenol blue, 50 mM Tris,

pH 6.8. Proteins were separated by SDS PAGE and either stained with Coomassie blue (90% of the immunoprecipitated protein) or subjected to Western blot analysis (remaining 10%) to make sure that the protein used for microsequencing had the correct molecular weight and that it was recognized by the myosin I β antibody. Those steps ensured that the correct protein was identified by use. Microsequencing was then performed using the microcapillary HPLC-electrospray ionization-tandem mass spectroscopy method (Hunt *et al.*, 1992). Three rounds of sequencing revealed slightly over 40% of the amino acid sequence.

<u>Results</u>: The state-of-the-art method for identifying a protein is to immunoprecipitate the protein using a specific antibody and to then microsequence the protein. The sequence obtained can then be compared with known protein sequences to positively identify the protein. Therefore, affinity purified antibodies to adrenal myosin I and standard methods were used to immunoprecipitate the 120 kD protein from the nucleus. These initial efforts using non-denaturing conditions to extract nuclei were unsuccessful because the antibodies did not immunoprecipitate the 120 kD protein. Therefore, the nuclear extracts were partially denatured and then renatured before the 120 kD protein was immunoprecipitated.

Using this method, the 120 kD protein was immunoprecipitated from nuclei isolated from NIH 3T3 cells, a mouse fibroblast cell line. SDS page showed the presence of a 120 kD band by Coomassie blue staining. A western blot confirmed that the 120 kD band in the immunoprecipitate was recognized by the myosin I β antibody described herein. Microsequencing of the 120 kD Coomassie blue stained band showed that the 120 kD protein has high sequence homology with myosin I β showing it to be an isoform of that protein (FIG. 1). It also revealed the presence of a 12 amino acid N-terminal extension that is unique to this protein (FIG 1).

With the exception of the N-terminal extension, the sequences identified by microsequencing (FIG. 1, underlined) are identical to the amino acid sequence of mouse cytoplasmic myosin I β. The NMI β contains at least part of the consensus ATP (brackets) and actin (italics) binding sites (FIG. 1) because the micro-sequencing overlapped both of these highly conserved sites. In FIG. 1, the consensus start site methionine is identified with an asterisk and the unique peptide is in bold.

The data presented herein demonstrate the following. (a) The presence of a 120-kD protein in nucleus that is recognized by polyclonal antibodies to adrenal myosin I β. (b) Immunostaining of the nucleus with affinity-purified polyclonal antibodies to adrenal myosin I. (c) Co-localization of the 120-kD protein and actin in the nucleus. (d) The presence of K^+-EDTA ATPase activity in column fractions containing the 120-kD protein following gel filtration chromatography of a nuclear extract. (e) Actin binding by the 120-kD protein only in the absence of ATP. (f) Positive identification of the 120 kD protein as a NMIβ protein; (g) proof NMIβ contains a unique N-terminal extension.

B. CHARACTERIZATION OF THE cDNA OF NUCLEAR MYOSIN I

Following the demonstration that the 120 kD protein is a previously undescribed myosin I β isoform present in the nucleus, the function of this protein was analyzed. Unexpectedly, a 16 amino acid N-terminal extension was found for that protein that distinguished the sequence from cytoplasmic myosin I β. This unique 16 amino acid N-terminal extension has two surprising characteristics. First, it is essential for nuclear localization of the protein. Second, it is involved in transcription when acting with RNA polymerase II.

1. PCR Analysis to Obtain cDNA for the N-terminal Extension

Ins A1

A 5' RACE was performed using a Mouse Marathon-Ready adapter ligated embryonic mouse cDNA library (Clontech Laboratories, Palo Alto, CA). A myosin I primer (5'-CAGGAGGTAACTGAGAATGTGG-3') that anneals 571 bases downstream from the N-terminal end of the gene was used in combination with an AP-1 (adapter primer 5'-CCATCCTAATACGACTCAC TATAGGGC-3'). PCR was performed using the Advantage cDNA Polymerase mix (Clontech Labs, Palo Alto, CA).

Two bands were obtained. One corresponded to the expected 600 base pair fragment and the other to a longer fragment of about 800 base pairs. Cloning and sequencing showed that the smaller band corresponds to the known mouse myosin I β. The bigger fragment had an N-terminal extension not previously found in myosin I β. Most importantly, 12 of the 16 amino acids in the 5' extension were also present in one of the peptides that was microsequenced (FIG. 2).

In FIG. 2, the two ATG's corresponding to the consensus start site and the predicted NMI start site are underlined. The peptide that overlaps the consensus start site that was obtained by microsequencing (ASALGSDGVRVTMESALTAR) is shown in bold while the known mouse myosin I β cDNA and protein sequences are in italics. The primer used for 5' RACE was 571 base pairs downstream of the consensus myosin I β start site.

Thus, this new isoform of Myosin I β contained a unique 16 amino acid N-terminal extension. This unique 16 amino acid extension at the N-terminus of Nuclear Myosin I differentiates it from Myosin I β found in the cytoplasm.

2. Domain Structure of the Mouse Myosin I β Gene

The mouse myosin I β gene is located in chromosome 11. It is about 12 kb long and contains 13 exons, starting with Exon 1 (Hamilton *et al.*, 1997). The mRNA transcripts obtained from the PCR analysis described above were from the same gene on chromosome 11 for the following reasons. First, none of the mRNA's contained sequences that differ from the myosin I β gene on chromosome 11. Second, Fluorescence in Situ Hybridization (FISH) mapped the myosin I β gene to a single locus in chromosome 11 (Hasson, *et al.*, 1996). Third, a single cDNA was present in adult rats that contained 2 potential start sites (Ruppert *et al.*, 1995). Although this rat (myr 2) cDNA was very similar to the NMI β cDNA, only a single protein that starts at the consensus start site was reported (Ruppert *et al.*, 1995).

Analysis of the mouse myosin I β gene uncovered 2 additional exons (Exon A and B) located upstream of Exon 1(Pestic-Dragovich *et al.*, 2000). Initial analysis of the 3' RACE PCR revealed the presence of about 1000 bases in the non-translated region that were not found in the mouse myosin I β cDNA's (Hamilton *et al.*, 1997; Crozet *et al.*, 1997). Only about half of these exactly match the 3' UTR of the rat myosin I β cDNA (Ruppert *et al.*, 1995).

Although the protein-coding regions of nuclear myosin I β cDNA and mouse myosin I β were identical, the 5' untranslated regions were quite different. The NMI β cDNA isolated by 5' RACE PCR from an embryonic mouse library had an unique 5' region. This 42 nucleotide region at the 5' end of the NMI β mRNA was from Exon B. Exon -1 contained a second translational start codon that was in the same reading frame with the consensus myosin I β translational start site on Exon -1.

Translation starting from the first ATG on Exon B produced a myosin I β with 16 extra amino acids at the N-terminal. Of these, 6 amino acids come from Exon -1 and 10 come from Exon 1. Exon -1 also contributed a stretch of 24 nucleotides, very rich in GC, that constituted the 5' UTR of NMI β. Therefore, the cytoplasmic and nuclear myosin I β isoforms were translated from separate transcripts from the same gene on chromosome 11.

3. Nuclear Localization of the FLAG Epitope

__**Procedure:**__ The NMIβ and CMIβ cDNA's were cloned into the pCMV-tag vector (Strategene, La Jolla, CA) that contained the FLAG epitope. NMIβ cDNA obtained by 5' RACE PCR was cut with EcoR I at the 3' end 9 nucleotides upstream of the stop codon and ligated and cloned into EcoR I cloning site of the pCMV-Tag 4 vector (Stratagene, La Jolla, CA). This vector had staggered ends and DNA sequencing was used to determine which of the NMIβ cDNA constructs was in the proper reading frame with the FLAG epitope. Cytoplasmic myosin I β (CMI β) cDNA was also ligated and cloned into pCMV-Tag 4 vector using the same procedure.

3T3 cells growing on coverslips were transfected with 3 μg of FLAG tagged NMI β or CMI β cDNA using 10 μl of Lipofectamine reagent (Life Technologies, Rockville, MD). The cells were allowed to grow for 72 hours, fixed in 3% paraformaldehyde and permeabilized with 0.1% deoxycholic acid and 0.1% Triton X-100 in PBS. The cells were then stained with a monoclonal mouse anti-FLAG IgG (Stratagene, La Jolla, CA) for 2 hrs at room temperature and visualized using a Texas red conjugated secondary antibody. Coverslips were mounted using Vectashield (Vector Labs, Burlingame, CA with DAPI and cells were photographed on Zeiss Laser confocal microscope.

__**Results:**__ Confocal images of cells lipofected with the NMI β-FLAG plasmid showed staining of both the nucleus and the cytoplasm. In contrast, the FLAG epitope was confined to the cytoplasm in cells lipofected with the CMI β-FLAG plasmid. A Z-stack, a three-dimensional image generated using confocal microscopy, confirmed the nuclear localization of the NMI β-FLAG and cytoplasmic localization of the CMI β-FLAG. There were two reasons for the nuclear and cytoplasmic staining in cells transfected with the NMIβ-FLAG. First, NMIβ had to

be synthesized in the cytoplasm and then transported to the nucleus. Second, the NMIβ cDNA contained start sites for both the NMIβ and CMIβ. Consequently, both proteins, each tagged with the FLAG epitope, were translated and resulted in staining of both the nucleus and the cytoplasm.

Thus, expression of the cDNA for the nuclear isoform of myosin I β (NMIβ) resulted in nuclear localization of an epitope tag. In contrast, expression of the shorter myosin I β isoform (CMI β) resulted in cytoplasmic localization of the epitope tag exclusively.

4. Production and Partial Characterization of an Anti-NMI β Antibody

a. Polyclonal Antibodies to NMIβ

Procedure: To produce polyclonal antibodies to the 16 amino acid N-terminal extension of NMI β, the peptide was synthesized followed by 3 glycine residues and a cysteine residue at the C-terminal end. The glycines residues were used to give rotational freedom to the peptide while the cysteine residue was used to attach the peptide to Keyhole Limpet hemocyanin (KLH), thyroglobulin and bovine serum albumin. Rabbits were immunized with 25 μg, each, of the peptide-KLH conjugate emulsified in complete Freund's adjuvant. The rabbits were boosted 4 weeks later with 25 μg, each, of the peptide-thyroglobulin conjugate emulsified in incomplete Freund's adjuvant. Immunizing rabbits with different conjugates limited the production of antibodies to the carrier. The rabbits were then bled once a week starting 10 days after the boost. All procedures were performed with prior approval from the University of Illinois at Chicago Animal Care Committee and with the supervision of University of Illinois at Chicago Veterinary Staff. Specific, anti-peptide antibodies were purified by applying antisera to an affinity column made by coupling the peptide-BSA conjugate to Sepharose 4B.

Results: For a microscopy evaluation, 3T3 cells were stained with affinity purified anti-NMI β peptide antibodies or the same antibodies pre-adsorbed with the peptide-KLH conjugate. Confocal microscopy showed that the antibodies stained the cytoplasm and the nucleus, consistent with the idea that the NMI β is synthesized in the cytoplasm and then transported into the nucleus. Equally

importantly, there was no staining when the antibodies were adsorbed with the KLH-NMI β peptide conjugate. Thus, these data unexpectedly showed that the 16 amino acid extension was responsible for directing the NMI β to the nucleus.

b. Monoclonal Antibodies

Antibodies that are produced by hybrid cells or hybridomas are called monoclonal antibodies. The hybrid cells are produced by fusing an antibody-secreting cell isolated from an immunized animal with a myeloma cell, which is a type of B-cell tumor. Monoclonal antibodies are powerful reagents for testing for the presence of a desired epitope because all of the antibodies produced by hybridomas detect the same epitope. A technique for production of monoclonal antibodies is known in the art and is described by Harlow *et al.* (1988). There are three stages to production of monoclonal antibodies. First, the animals must be immunized by injecting the animal with an antigen preparation. Second, a screening procedure must be developed where the sera from test bleeds are used to develop and validate it. Finally, the hybridomas can be produced by first boosting the animals with a sample of the antigen several days prior to fusion. Then, the immunized animal is used to prepare antibody secreting cells which are then mixed with myeloma cells and fused. The cells from positive cells are grown and single-cloned to produce the monoclonal antibodies.

5. Co-Localization of NMI β and RNA Polymerase II

Procedure: HeLa cells were grown at 37°C in suspension in Eagle medium containing 5% FBS. The culture was split into three portions and inhibitors of RNA transcription were added to the second (20 μg/ml α-amanitin for 4 hr, 40 min.) and third (0.5 μg/ml actinomycin D for 1 hr) portions. After the desired inhibition time, cells were pelleted, fixed in 3% paraformaldehyde plus 0.1% glutaraldehyde in Sörensen buffer, embedded and sectioned using methods known to those of skill in the art (Pestic-Dragovich *et al.*, 2000). Eighty nanometer thick sections were cut and simultaneously incubated with a monoclonal antibody that recognized the largest subunit of RNA polymerase II (Lavoie *et al.*, 1999) and with the rabbit polyclonal antibody to adrenal myosin I (both 10 μg/ml). The cells were then washed and incubated with 5 nm gold-conjugated goat anti-mouse antibody and

with 10 nm gold-conjugated goat anti-rabbit. After washing, the sections were contrasted for 5 min with a saturated solution of uranyl acetate in water

<u>Results</u>: Confocal and electron microscopy revealed co-localization of NMI β and RNA polymerase in control cells (Pestic-Dragovich *et al.*, 2000). The co-localization of NMI β and RNA polymerase II was lost when cells were treated with α-amanitin, which blocks transcription by stimulating the degradation of the large subanit of RNA polymerase II (Nguyen *et al.*, 1996). The absence of co-localization was shown in two ways: By a reduced density of RNA polymerase II labeling and insignificant co-localization of labels. The co-localization decreased when cells were treated with actinomycin D, which blocks transcription by binding to DNA (Alberts *et al.*, 1994). The decrease was shown by the rarity of the co-localization of RNA polymerase II with NMI β. When control samples were incubated as above but with one of the primary antibodies omitted, they showed no significant gold labeling.

6. Co-precipitation and In Vitro Transcription Assays.

<u>Procedure</u>: NMI β was immunoprecipitated from nuclear extracts using affinity purified antibodies to the NMIβ peptide or the same antibodies pre-adsorbed with the peptide. The immunoprecipitates were analyzed by protein immunoblotting using anti-NMI β peptide antibodies or an antibody to the large subunit of RNA polymerase II (Lavoie *et al.*, 1999).

An *in vitro* transcription assay that quantifies transcription initiation by RNA polymerase II was performed using a HeLa nuclear extract system as described by the manufacturer (Promega, Madison, WI). Nuclear extract (8 units) treated with RNAguard RNase inhibitor (Pharmacia, Piscataway, NJ) was preincubated with buffer, affinity purified antibodies to smooth muscle myosin II (de Lanerolle *et al.*, 1980), anti-NMI peptide antibody or 0.5 μg α-amanitin (30 mins at room temperature). HeLa cell template DNA (100 ng) containing the cytomegalovirus immediate early promoter was added and the reaction mixture (25 μl) was made with 3 mM $MgCl_2$, 0.4 mM, each, ATP, CTP and UTP. Unlabelled GTP (0.04 mM) and[α^{32}P]-labelled GTP (10 μCi) was then added and the reaction mixtures were incubated for 30 mins at 30°C. The transcription products were isolated and separated by 6% acrylamide, 7 M urea denaturing gel electrophoresis and analyzed using a phosphorimager.

Results: Western blotting revealed the presence of RNA polymerase II in the NMI immunoprecopitate when the blots were probed with an antibody to RNA polymerase II (FIG. 4A). RNA polymerase II was not present in the immunocomplexes when the nuclear myosin I β antibody was pre-incubated with the peptide (FIG. 4A). In addition, the anti-NMIβ-peptide antibody inhibited RNA synthesis, in vitro (FIG. 4B). In contrast, neither the same antibodies pre-incubated with peptide nor affinity purified antibodies to myosin II inhibited transcription (FIG. 4B).

7. Methods for Screening for Agents or Procedures that inhibit NMI Expression or Function .

Because NMI forms a complex with RNA polymerase II and is required for transcription as shown above, agents or procedures that inhibit the expression or functions of NMI could inhibit transcription. A methods for inhibiting NMI expression could include antisensense oligonucleotides (Toulme, 2001). Antisense oligonucleotides have been used over the past 15 years to regulate the expression of specific genes by RNA targeting. The presence of a unique, N-terminal extension in NMI that is not found in other proteins makes NMI an ideal target for antisense oligonucleotides. Oligonucleotide sequences (16 to 20 nucleotides long) that are complementary to the coding region for the unique peptide could be administered to cells. These oligonucleotides could be synthesized by commercial vendors and derivatized, using methods familiar to those skilled in the art, to enhance cellular entry and to enhance binding to RNA. The effects on NMI expression can be monitored by performing western blot analyses or immunofluorescence microscopy, using polyclonal antibodies to adrenal myosin I, polyclonal antipeptide antibodies to NMI or monoclonal antibodies to NMI followed by an appropriate fluorescently-labelled secondary antibody, as described above. The absence of a band that co-migrates with NMI found in nuclear extracts or the absence of nuclear staining by one of these antibodies would provide proof for the suppression of NMI expression.

A method for screening for agents that inhibit NMI function is also of utility. Such agents could include naturally occurring or synthetic compounds, drugs or other formulations that form a complex with NMI and inhibit the translocation of NMI to the nucleus, inhibit the enzymatic activity of NMI and/or the binding of NMI to RNA

polymerase II. In such a screening method, cells would be plated on cover slips in media containing 10% calf serum. The cells would then be synchronized by growing them for 24-48 hours in media not containing calf serum, a method familiar to those skilled in the art. The cells would then be stimulated to enter the cell cycle by adding media containing 10% serum and treated with one of the said agents for a period of time. The cells would then be fixed and permeabilized as described above.

Agents that prevent the translocation of NMI β to the nucleus can be detected by staining cells with using polyclonal antibodies to adrenal myosin I, polyclonal anti-peptide antibodies to NMI or monoclonal antibodies to NMI β followed by an appropriate fluorescently-labelled secondary antibody, as described above. A decrease or an absence of nuclear staining would indicate inhibition of translocation of NMI β to the nucleus.

Agents or procedures that inhibit NMI translocation or function should also inhibit transcription. To detect inhibition transcription, cells are grown at 60-80% confluency on polylysine or fibronectin coated coverslips so that cells will not be washed away in the permealization step. The cells are rinsed once in glycerol buffer [5 mM $MgCl_2$, 25% glycerol, 0.5 mM PMSF (add fresh), 0.5 mM EGTA, 20 mM Tris-HCl, pH 7.4] at room temperature and permeablized in the glycerol buffer + 5 μg/ml digitonin (Calbiochem, La Jalla, CA) for 3 mins at room temperature. The permealization buffer is removed and the transcription mixture [100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 25% glycerol, 1 mM PMSF (add fresh), 2 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.2 mM Br-UTP (Sigma, St. Louis, MO), 100 mM, 1 uL/ml RNAsin (add fresh), 50 mM Tris HCl, pH 7.4.] is added. The cells are then incubated at 37 degrees centigrade as desired (7-10 mins). At the end of transcription, the cells are rinsed very gently 2X in PBS and then immediately fixed in 3% freshly made formaldehyde in PBS for 7 mins at room temperature. Cells are then permeabilized with 0.1% deoxycholic acid and 0.1% Triton X-100 in PBS for 7 mins at room temperature and stained with a Alexa Flour-conjugated mouse moloclonal antibody to BrUTP (Molecular Probes, Eugene, OR) for 2 hrs at room. The coverslips are washed and mounted using Vectashield (Vector Labs, Burlingame, CA) with DAPI and the cells are examined and photographed with Zeiss Laser confocal microscope using the appropriate filters.

DOCUMENTS CITED

Alberts, B. *et al.* *The Molecular Biology of the Cell, 3rd Edition*, Garland Publishing Co., New York, 1994, pp. 240.

Barylko, B., Wagner, M.C., Reizes, O. and Albanesi, J.P. Purification and characterization of a mammalian myosin I. *Proc. Natl. Acad. Sci. USA* 89, 490-494, 1992.

Berezney, R. and Coffey, D.S. Nuclear matrix protein: association with newly syn-thesized DNA. *Science* 18, 291-293, 1975.

Cook, P.R. The nucleoskeleton and the topology of replication. *Cell* 66, 627-635, 1991.

Cook, P.R. The organization of replication and transcription. *Science* 284, 1790-1795, 1999.

Crozet, F. . . and Petit, C. Cloning of the genes encoding two murine and human cochlear unconventional type I myosins. *Genomics* 40, 332-341, 1997.

de Lanerolle, P. and Stull, J.T. Myosin phosphorylation during contraction and relaxation of tracheal smooth muscle. *J. Biol. Chem.* 255, 9993-10000, 1980.

Hamilton, B.A. *et al.* The vibrator mutation causes neurodegeneration via reduced expression of PITP alpha: positional complementation cloning and extragenic suppression. *Neuron* 18, 711-22, 1997

Harlow, E., Lane, D.P. Antibodies: A laboratory manual.139-243, 1988.

Hasson, T., Skowron, J.F., Gilbert, D.J., Avraham, K.B., Perry, W.L., Bement, W.M., Anderson, B.L., Sherr, E.H., Chen, ZY., Green, L.A., Ward, D.C., Corey, D.P., Mooseker, M.S., Copeland, N.G., and Jenkins, N.A. Mapping unconventional myosins in mouse and human. *Genomics* 36, 431-439, 1996.

Hunt, D.L. *et al.* Characterization of peptides bound to the Class 1 MHC molecule HLA-A2.1 by mass spectroscopy. *Science* 255, 1261-1263, 1992.

Lavoie, S.B., Albert, A.L., Thibodeau A. and Vincent, M. *Biochem Cell Biol.*, 77, 367-74, 1999.

Mermall, V., Post, P.L and Mooseker, M.S. Unconventional myosins in cell movement, membrane traffic and signal transduction. *Science* 279, 527-533, 1998.

Nguyen, V.T., Giannoni, F., Dubois, M.F., Seo, S.J., Vigneron, M., Kédinger, C. and Bensaude, O. In vivo degradation of RNA polymerase II largest subunit triggered by α- amanitin. *Nucleic Acids Res* 24, 15,292-15,929, 1996

Nowak., G., Pestic-Dragovich, L., Hozak, P., Philimonenko, A., Simerly, C., Schatten, G. and de Lanerolle, P. Evidence for the presence of a myosin I like protein in the nucleus. *J.Biol. Chem.* 272, 17,176-17,181, 1997.

Pestic-Dragovich, L., Stojiljkovic, L., Philimonenko, A., Nowak, G., Ke, Y., Settlage, R.E., Shabanowitz, J., Hunt, D.F., Hozak, P. and de Lanerolle, P. A myosin I isoform in the nucleus. *Science* 290: 337-341, 2000

Pollard, T.D. and Korn, E.D. Acanthamoeba myosin I. Isolation from Acanthaamoeba castellanii of an enzyme similar to muscle myosin. *J. Biol. Chem.* 248, 4682-4690, 1973.

Rando, O.J., Zhao K. and Crabtree, G.R. *Trends in Cell Biol.* 10, 92-97, 2000.

Ruppert, C., Godel, J., Muller, R.T., Kroschewski, R., Reinhard, J. and Bahler, M. Localization of the rat myosin I molecules myr 1 and myr 2 and in vivo targeting of their tail domains. *J. Cell Sci.* 106, 3775-3786, 1995.

Scheer, U. and Hinssen, H., Franke, W.W. and Jokusch, B.M. Microinjection of actin-binding proteins and actin antibodies demonstrates involvement of nuclear actin in transcription of lampbrush chromosomes. *Cell* 39, 111-122, 1984.

Toulme, J-J. New candidates for true antisense. *Nature Biotechnology* 19, 17-18, 2001.

Yin, H., Wang, M.D., Svoboda, K., Landick, R., Block, S.M. and Gelles, J. Transcription against an applied force. *Science* 270, 1653-1656, 1995.